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DECLARATION OF DR. ROBERT STERN UNDER 37 C.F.R. § 1.132	Attorney Docket Confirmation No.	UCSF-088 CON2 4596
	First Named Inventor	R. Stern
	Application Number	10/622,283
	Filing Date	July 18, 2003
	Group Art Unit	1652
Address to: Commissioner for Patents Alexandria, VA 22313-1450	Examiner Name	K.H. Gebreyesus
	Title	*Human plasma hyaluronidase*

Dear Sir:

1. I, Robert Stern, declare that I am a co-inventor of the claims of the above-identified patent application. I directed others and/or personally performed the research leading to the invention disclosed and claimed therein.

2. I have read the Office Action dated May 18, 2005 in this application and understand that the Examiner has rejected pending claims 34-38 and 40-58 on the basis that the subject matter of the claims was considered to by disclosed by Afify et al. ((1993) *Arch. Biochem. Biophys.* 305:434-441; "Afify").

3. I am the corresponding author on Afify. I also supervised the research described in Afify. The work described in Afify was performed principally by a medical student who spent a summer in my laboratory.

4. The May 18, 2005 Office Action stated that Afify discloses the purification of hyaluronidase, from fresh human serum as starting material, to apparent homogeneity. The May 18, 2005 Office Action also indicated that the specific activity of the hyaluronidase of Afify is expressed

in a manner that is different from the expression of specific activity in the instant application. These issues are discussed below.

Human serum hyaluronidase was not purified to apparent homogeneity, as asserted by Afify; instead, human serum hyaluronidase represented less than 1% of the total protein in the preparation identified by Afify as purified human serum hyaluronidase.

5. Afify states that human serum hyaluronidase was purified from serum to homogeneity using a two-step procedure. Afify, page 434, Abstract; page 435, "Preparation of serum samples"; and page 436, bridging paragraph, columns 1 and 2. This conclusion was based upon the data depicted in Figure 3 of Afify. The protein preparation discussed in Figure 3 of Afify was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); and the gel was stained with Coomassie blue. A band that migrated with a relative molecular weight similar to that of a protein capable of degrading hyaluronan in SDS PAGE substrate gels was observed. Afify concluded that this band was purified human serum hyaluronidase. Unfortunately, this conclusion was made without knowledge of the actual concentration of hyaluronidase found in human serum, and was incorrect, as discussed further below.

6. The amount of hyaluronidase in human serum or human plasma is approximately 5.9 +/- 1.2 relative turbidity reducing units (TRU)/mL. Frost and Stern, *Anal Biochem.* 1997 Sep 5;251(2):263-9; a copy of which is provided herewith as Exhibit 2. The Frost and Stern method is the same as that described in the instant application. Instant application, page 51, line 1 to page 52, line 10. Other assays utilizing different methodologies have shown that both human serum and plasma have relatively constant levels of hyaluronidase activity. Natowicz et al., *Clin Chim Acta.* 1996 Feb 9;245(1):1-6; a copy of which is provided herewith as Exhibit 3.

7. The method that Afify used for determining hyaluronidase activity differs from the method used in the instant application. Afify used an earlier method for measuring

hyaluronidase activity. Stern and Stern, *Matrix*. 1992 Nov;12(5):397-403; a copy of which is provided herewith as Exhibit 4. The hyaluronidase activity assay used by Afify differs in at least two important ways from the Frost and Stern method. First, in the Afify method, the substrate was detected indirectly with a biotinylated hyaluronan binding probe, while the Frost and Stern method involves use of a directly biotinylated substrate. Secondly, Afify used a *Streptomyces* hyaluronidase enzyme standard, while Frost and Stern used a bovine testes sources hyaluronidase standard Wydase®. The *Streptomyces* hyaluronidase standard is calibrated at a different pH than the bovine hyaluronidase standard and results in a relative Unit conversion of approximately 6 (Stern and Stern) Units for every (Frost and Stern) Unit. However, as discussed below, this conversion is not required to evaluate the purity of the preparations described by Afify and those described in the instant application.

8. A comparison of Afify's purification process and the purification method described in the instant application highlights the differences in the outcome. The Table presented below summarizes the data and calculations provided in Afify and in the instant application. Afify started with approximately 1.2 mL of human serum. Afify, page 435, "Preparation of serum samples." The instant application describes a working example (Example 2) starting with approximately 2100 mL of human plasma. Instant application, page 52, line 11 to page 54, line 24. It is important to note that human serum and plasma have approximately the same amount of relative hyaluronidase activity in both assays. Afify asserts a 5,733-fold purification (defined as the Final Specific activity divided by the Starting Specific Activity); a calculated 800% recovery of total activity (calculated as: (Total Activity End ÷Total Activity Starting) x 100); and a recovery of 0.144 mg protein from 103 mg total starting protein. In comparison, Example 2 of the instant application describes a purification scheme that resulted in a 1,488,889-fold purification; an 18% recovery of total activity; and a recovery of 0.0225 mg protein from 180,600 mg starting protein. Afify, page 439, Table 1; and instant application, Table 1, page 54. However, Afify describes the removal of an inhibitor in the purification process to account for the 800% recovery, effectively inflating the fold purification. Afify, page 437, column 2, second full paragraph.

Starting Material	Total Protein (mg)	Starting Activity U/mL	Starting Specific Activity (Units/mg)	Total Activity Starting (Units)	Total Protein End (mg)	Total Activity End (Units)	Final Specific Activity (U/mg)	Fold Purification Finish/Start
Afify et al human serum, 1.2mL	103	0.80	0.0093	0.96	0.1440	7.68	53.33	5,733
Frost et al, human serum 2100 mL	180,600	4.99	0.0580	10,475	0.0225	1,943	86,356	1,488,889
						Fold Difference		260
						Percent Hyaluronidase (Afify et al)		0.39%

9. The amount of human hyaluronidase present in 2100 mL of plasma based upon Example 2 of the instant application would therefore be 0.120 mg of total hyaluronidase protein, assuming 100% recovery. Afify however, reports 0.144 mg of hyaluronidase protein in the purified preparation from about 1.2 mL of human serum. Even accepting the presence of an inhibitor in the Afify preparation, there is a 260 fold difference in Fold Purification between Afify and the instant application.

10. Using the above-noted calculations of the Fold Purification from the instant application and from Afify, the actual amount of hyaluronidase present in the 1.2 mL of serum Starting Material used by Afify contained only 0.0000704 mg (ie 70 ng) of hyaluronidase protein. Thus, at most, 0.39% of the 0.144 mg of protein obtained by Afify, after purification by DEAE chromatography and gel filtration, could be the hyaluronidase enzyme, assuming 100% recovery of the enzyme and the presence of an inhibitor. Thus although the protein preparation reported in Afify exhibited hyaluronidase activity, using the sensitive method of substrate gel electrophoresis, hyaluronidase protein present in the protein preparation was in such low abundance that it would not be detectable by Coomassie Blue staining, even if the entire maximal 70 ng of protein were loaded on the SDS polyacrylamide gel.

The protein preparation asserted by Afify to be purified human serum hyaluronidase consists mostly of human serum albumin.

11. The protein immunopurified from human plasma using the method described in the instant application has N-terminal and internal amino acid sequences of human plasma hyaluronidase. However, N-terminal amino acid sequencing of the Afify protein preparation revealed a sequence identical to human serum albumin, the dominant protein present in human serum and plasma. The fact that Afify's protein preparation was identified as albumin is not surprising, given the fact that, as discussed above, the amount of hyaluronidase present in Afify's preparation was too low to be detected in a Coomassie Blue stained SDS polyacrylamide gel or by N-terminal amino acid sequencing. In view of the fact that the relative molecular weight of human serum albumin is similar to that of human plasma hyaluronidase, the mistaken identification of human serum albumin in the protein gel with the enzymatic clearing from trace amounts of human plasma hyaluronidase by substrate gel zymography is not entirely unexpected.

Conclusion

12. Human serum hyaluronidase was not purified to apparent homogeneity, as asserted by Afify; instead, human serum hyaluronidase represented less than 1% of the total protein in the preparation identified by Afify as purified human serum hyaluronidase. Human plasma hyaluronidase is present in human serum at concentrations that are too low to give rise to the amount of serum hyaluronidase asserted by Afify from only 1.2 ml serum. The SDS-PAGE band asserted by Afify to be highly purified human serum hyaluronidase is considered to consist mostly of albumin, which is present at high concentrations in human serum.

13. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

11/15/05
Date

Robert Stern
Robert Stern

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